Plaque Formation with Influenza Viruses in Dog Kidney Cells

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SUMMARY

Infectious influenza and parainfluenza viruses were produced as plaque-forming units (p.f.u.) in dog kidney cells (MDCK) after several passages of egg-grown viruses in MDCK monolayers. Virus titres of $10^6$ to $10^{11}$ p.f.u./ml were obtained for several A and B strains of influenza and parainfluenza viruses.

Plaque formation by influenza virus has been demonstrated in tissue cultures of Rhesus monkey kidney (Choppin, 1962) chick embryo fibroblast (Peters & Goldemans, 1966), chick embryo kidney (Maassab, 1968) and chick embryo lung (Stewart & Frickey, 1966). The disadvantage of the above-mentioned methods is the inconvenience of the primary cell cultures and their irregularity of plaque formation with different strains of influenza virus, particularly type A strains. Only three established cell lines were reported to support the replication of a variety of strains of influenza virus: (a) Chang conjunctiva line 1-5C-4 (Sugiura & Kilbourne, 1965); (b) G-2 (Hatano & Morita, 1967); and (c) MDCK-USD (Gaush & Smith, 1968). Except for the MDCK-USD system, however, most of these virus cell systems are limited to the synthesis of one or perhaps two strains of influenza virus.

In this paper, a plaque technique for influenza virus, using the commercially available parent dog kidney cells (MDCK) is described. Procedures to produce high yields of virus titres in p.f.u. are also presented for several strains of influenza virus.

Influenza A virus (A/PR/8/34) and influenza B virus (B/Maryland/1/59 and B/Hong Kong/73) were obtained from the National Institutes of Health. Para-influenza I (D/Sendai/52), influenza A virus (A1/Ann Arbor/1/57 and A2/Hong Kong/8/68) and MDCK cells were purchased from the American Type Culture Collection. The virus used in this study for the first passage in MDCK cells were grown in the allantoic cavity of ten-day-old embryonated chicken eggs. The eggs were inoculated with 0·2 ml of a tenfold dilution of each virus stock in phosphate-buffered saline (PBS) containing 50% bactobeeffinfusion and neopeptone (Difco).

After 48 h of incubation at 37 °C in a moist chamber, the allantoic fluid was harvested and clarified by centrifuging at 5000 g for 15 min. The virus was concentrated by centrifuging at 100000 g for 3 h and resuspension in one-tenth volume of PBS.

Influenza A virus (A1/Ann Arbor/1/57 and A/PR/8/34) and influenza B virus (B/Maryland 1/59) were inoculated at tenfold dilutions, while influenza A virus (A2/Hong Kong/8/68) and parainfluenza virus I (D/Sendai/52) were passed on to MDCK cells undiluted. Adsorption of virus was allowed to occur at 37 °C for 30 min. The monolayers were then overlaid with 5 ml of medium 199 containing 5% inactivated foetal calf serum and grown at 37 °C for periods between 48 and 72 h. Cultures were examined microscopically twice daily for degree of cellular aberration which included granulation, cell fusion and detachment of cells from cell sheets. All influenza virus strains performed in this study showed one of these cellular aberrations and were harvested at 48 h, while para-influenza virus I (D/Sendai/52) was harvested at 72 h. Crude virus extracts were prepared by rapid freezing and thawing of cell suspension twice and sonic treatment. Cell debris was removed by low-speed centrifugation at 100000 g for 2 h. The pelleted virus was suspended in 1·5 ml of PBS.
Table 1. Plaque number of influenza and para-influenza viruses after passages in MDCK cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of passages</th>
<th>p.f.u./ml</th>
<th>HA titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34</td>
<td>9</td>
<td>1.6 x 10^8</td>
<td>1/256</td>
</tr>
<tr>
<td>A1/Ann Arbor/1/57</td>
<td>11</td>
<td>1.4 x 10^11</td>
<td>1/1024</td>
</tr>
<tr>
<td>A2/Hong Kong/8/68</td>
<td>8</td>
<td>1.0 x 10^7</td>
<td>1/128</td>
</tr>
<tr>
<td>B/Hong Kong/73</td>
<td>9</td>
<td>3.5 x 10^5</td>
<td>1/28</td>
</tr>
<tr>
<td>B/Maryland/1/59</td>
<td>7</td>
<td>1.0 x 10^6</td>
<td>1/64</td>
</tr>
<tr>
<td>Parainfluenza I D/Sendai/52</td>
<td>7</td>
<td>2.0 x 10^5</td>
<td>1/32</td>
</tr>
</tbody>
</table>

* Crude virus extracts were prepared by rapid freezing and thawing and sonic treatment of infected monolayers of MDCK cells. After removal of cell debris by low-speed centrifugation, virus was concentrated by high-speed centrifugation and resuspension in one-tenth volume of PBS. The results are expressed as p.f.u./ml of the original volume of medium.

Beginning with the second passage, all virus suspensions were diluted tenfold for inoculation and the conditions for virus adsorption and growth were the same as described for the first tissue culture passage. Harvesting of virus was performed at 48 h, but only the medium from infected cells was passed on to the new cultures.

For plaque assays, confluent monolayers of MDCK cells were infected with 0.5 ml of appropriate dilutions of virus stocks in PBS. Adsorption of virus was allowed to occur at 37 °C for 1 h with frequent agitation. At the end of the adsorption period, monolayers were overlaid with 5 ml of medium 199 containing 5% foetal calf serum, 0.005% dimethylsulphoxide (DMSO) and 0.25% agar (Seakem grade, MCI Biomedical) and grown at 37 °C for periods between 72 and 96 h. Agar-covered monolayers were fixed in 10% formalin and 2% sodium acetate. After removal of agar, the cells were stained with 0.5% crystal violet solution and the plaques were scored by transmitted light. The addition of DMSO to agar overlays slightly improved the precision of plaque assays with influenza virus. The improvement is not as great as that observed with simian virus 40 (Cleaver, 1974) but for experimental consistency, DMSO was included in the agar overlay. All the A and B strains of influenza virus tested produced large sizes of plaques (3 to 4 mm in diam.), while smaller but discernible plaques were formed by para-influenza I (D/Sendai/52). A few smaller plaques, however, were frequently observed with A and B strains. This phenomenon seems to be due to secondary plaques, since the appearance of these smaller plaques depends on the concentration of agar used and time of incubation.

All strains of virus induced cytopathic effect (c.p.e.) at first tissue culture passage as characterized by cellular fusion, extreme granulation, rounding of cells and almost detachment of cell sheets at 48 h of incubation. Haemagglutination (HA) titres of 1/2 to 1/256 were obtained from first to third tissue culture passages. However, no p.f.u. were observed in these HA positive media. Nonetheless, HA titres increased slowly with each passage. By the fourth passage, infectious virus was demonstrated by plaque assay ranging from 10^4 to 10^8 p.f.u./ml. Table 1 shows the yield of infectious virus as p.f.u./ml from different strains of virus after several passages. Of the six strains of influenza virus tested, all produced reasonably good virus and HA titres after the seventh passage. A high titre of 10^{11} p.f.u./ml was obtained with influenza A virus (A1/Ann Arbor/1/57) when the passage was conducted to the eleventh time.

The infectious titre was the average result of three separate titrations. The titres varied from 10^9 to 10^{11} p.f.u./ml from the ninth passage on, and haemagglutination titres of 1/256 to 1/1024 were obtained, respectively, for influenza A virus (A1/Ann Arbor/1/57).
Table 2. Antigenic analysis of adapted influenza virus

<table>
<thead>
<tr>
<th>Antisera*</th>
<th>A1/Ann Arbor/1/57</th>
<th>A/PR/8/34</th>
<th>B/Maryland/1/59</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1/Ann Arbor/1/57</td>
<td>2048</td>
<td>1024</td>
<td>64</td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>4096</td>
<td>4096</td>
<td>324</td>
</tr>
<tr>
<td>B/Maryland/1/59</td>
<td>0</td>
<td>0</td>
<td>1024</td>
</tr>
</tbody>
</table>

* Antisera to A/PR/8/34 and B/Maryland/1/59 were obtained from the National Institute of Health. Antisera to A1/Ann Arbor/1/57 was prepared in chickens by standard methods used for immunizations.

† HI titres are the reciprocal of serum dilutions causing 100% inhibition of four haemagglutinating units.

The identities of the adapted viruses were confirmed by serological methods. Table 2 shows that while there is an asymmetrical cross-reaction between A1/Ann Arbor/1/57 and A/PR/8/34, B/Maryland/1/59 retains specificity toward its own antisera.

In conclusion, a plaque assay was established in MDCK cells for several strains of tissue culture adapted influenza viruses. High virus titres could be obtained after relatively few passages. If this result is applicable to other recently isolated influenza virus strains, it might provide a method for increasing the speed and convenience of influenza virus plaque assays and for genetic studies.

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REFERENCES


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